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MUCOSAL CYTOTOXIC T LYMPHOCYTE RESPONSES

Related Applications

The present application is a continuation-in-part application of, and claims the benefit under Title 35 of U.S. Provisional Application Nos. 60/058,523 filed on September 11, 1997, and 60/074,894 filed on February 17, 1998.

Technical Field

The present invention relates to methods and compositions for stimulating immune responses in mammals. More particularly, the invention relates to methods and compositions for stimulating mucosal immunity.

Background of the Invention

Many infectious pathogens, e.g., *HIV-1*, enter their mammalian hosts via a mucosal tissue prior to establishing a systemic infection. Veazey, et al., Science 280:427-431, 1998. Accordingly, vaccines capable of protecting against HIV should be capable of inducing long-term mucosal immune responses. A number of recent studies have shown that such immune responses require direct stimulation of mucosal tissues, and may be achieved with live attenuated virus, Cranage, et al., Virology 229:143-154, 1997, subunit SIV envelope Lehner, et al., Nature Medicine 2:767-775, 1996, HIV-recombinant viruses, including recombinant MVA 89.6 env (Belyakov et al., unpublished), or HIV peptide constructs Belyakov, et al., Proc. Nat. Acad. Sci. 95:1709-1714, 1998 (see also, Gallichan, et al., J. Exp. Med. 184:1879-1890, 1996; Cranage, et al., Virology 229:143-154, 1997; and Rosenthal, et al., Semin. Immunol. 9:303-314, 1997).

Numerous questions remain, however, concerning which vaccine candidates may afford the most effective protection against mucosal challenge with virus, and what

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mechanisms may be involved in mediating protective immunity. While a number of studies have shown a role for CTL in protection against infections such as influenza that have a mucosal component (Taylor and Askonas, Immunology 58:417-420, 1986; Epstein et al., J. Immunol. 160:322-327, 1998; Kulkarni et al., J. Virol. 69:1261-1264, 1995), these reports have not established whether the CTL need to be in a local mucosal site to protect. Conversely, while other studies have shown the induction of CTL in the mucosa, they have not established that these cells have a role in protection (Gallichan and Rosenthal, J. Exp. Med. 184:1879-1890, 1996; Bennink et al., Immunology 35:503-509, 1978; Lohman et al., J. Immunol. 155:5855-5860, 1995); and Klavinskis, et al., J. Immunol. 157:2521-2527, 1996 J. Immunol. 155:5855-5860, 1995. Yet other studies have shown the induction by vaccines of protective immunity in the mucosa, but in the face of multiple immune responses, have not been able to sort out which responses are involved in protection (Lehner et al., Nature Medicine 2:767-775, 1996; Putkonen et al., J. Virol. 71:4981-4984, 1997; Miller et al., J. Virol. 71:1911-1921, 1997; Quesada-Rolander et al., AIDS Res Hwn Retroviruses 12:993-999, 1996; Bender et al., J. Virol. 70:6418-6424, 1996; Wang et al., Vaccine 15:821-825, 1997).

Thus, although the role of CTL in protection against mucosal infections has been of interest for decades, especially in the case of influenza virus, prior investigations have failed to identify fundamental mechanisms linking immune responses to protection. In this regard, because mucosal infection by virus induces a local IgA response, it has been too readily assumed that this response, and not a concomitant CTL response, was responsible for protection against viral infection through the mucosal route. However, the role of secretary IgA in neutralizing and protecting against mucosal HIV challenge is also not clear.

CTL are crucial mediators of immunity to intracellular microorganisms such as viruses as well as certain bacteria and protozoan parasites. CTL specifically recognize "non-self" antigenic peptides bound to major

histocompatibility complex (MHC) class I molecules on the surface of "target cells" and then kill the target cells expressing the non-self antigenic peptides. Non-self polypeptides from which the non-self peptides are derived can be a) proteins encoded by intracellular microbes, b) host-encoded proteins whose expression is induced by a microbe, or c) mutant host encoded proteins expressed by, for example, tumor cells.

Thus, generation of CTL responses in the inductive and the effector mucosal immune system may be important to establishing effective protective immunity to intracellular microbial pathogens that establish infection via the mucosal barriers. In some cases, administration of antigens via parenteral routes (subcutaneous, intramuscular, intravenous or intraperitoneal, for example) either fails to induce mucosal immunity or does so extremely inefficiently.

As noted above, previous reports of mucosal immune responses elicited by mucosal challenge with viruses have disclosed that the latter induces antiviral antibody responses, and in some cases CTL responses, in the intraepithelial lymphoid populations. Chen et al., _J. Virol._ _71_:3431-3436, 1997; Sydora, et al., _Cell Inununol. 167_:161-169, 1996. However, it is not clear if either of such responses is relevant to protection against viral infection in general or HIV infection in particular. Additional studies have suggested a role for CTL in protection against infections that involve the mucosa, such as influenza or respiratory syncytial virus, Taylor et al., _Immunology 58_:417-420, 1986; Epstein et al., _J. Immunol. 160_:322-327, 1998; Kulkarni et al., _J. Virol. 69_:1261-1264, 1995. However, these studies have not addressed the question of whether CTL must be present at the mucosal site of infection, or if their principal activity occurs systemically.

Accordingly, a need exists in the art to better define the roles and mechanisms of CTL in mediating immunity and to develop new tools for mediating immune protection against HIV and other pathogens, particularly by conferring

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immune protection at mucosal sites where such pathogens
initially proliferate.

Summary of the Invention

    The present invention is directed to methods and
compositions for inducing a protective mucosal CTL response in
a subject. The methods of the invention involve administering
either a soluble antigen itself, or a polynucleotide encoding
the soluble antigen, to a mucosal surface. The soluble
antigens can be full length, naturally occurring polypeptides
or fragments (i.e., peptides) derived from them. Peptides to
be administered can be any length less than that of the
naturally occurring polypeptide. They can be, for example,
five to one hundred amino acid residues long, preferably
twenty to seventy five amino acid residues long, more
preferably twenty five to sixty amino acid residues long and
most preferably thirty to fifty amino acid residues long.

    The soluble antigen is administered with an
adjuvant at the mucosal site or without an adjuvant.
Adjuvants can be, for example, cholera toxin (CT), mutant CT
(MCT), E. coli heat labile enterotoxin (LT) or mutant LT
(MLT). IL-12 and/or IFNγ can be administered with the soluble
antigen either in the presence or absence of an adjuvant.
Alternatively, the two cytokines (IL-12 and/or IFNγ) can be
administered systemically and separately from the soluble
antigen which is administered mucosally, optionally with
adjuvant. Mucosal routes of administration include IR,
intranasal (IN), intragastric (IG), intravaginal (IVG) or
intratracheal (IT).

    Soluble antigens can be derived from pathogenic
viruses (e.g., *HIV-1*, influenza virus or hepatitis A virus),
bacteria (e.g, *Listeria monocytogenes*), protozoans (e.g.,
Giardia lamblia). Alternatively, the soluble antigen can be a
tumor-associated antigen, e.g., prostate specific antigen
produced by prostate tumor cells or tyrosinase produced by
melanoma cells. Peptide antigens can be cluster peptide
vaccine constructs (CLUVAC). For example, an *HIV-1* CLUVAC can
include one or more of the following sequences:
EQMHEDIISLWDQSLKPCVKRIQRGPGRAFVTIGK (SEQ ID NO:1),

KQIINMWQEVGKAMYAPPISGQIRRIQRGPGRAFVTIGK (SEQ ID NO:2),
RDNWRSELYKYKVVKIEPLGVAPTRIQRGPGRAFVTIGK (SEQ ID NO:3),
AVAEGTDRVIEVVQGAYRAIRHIPRRIRQGLERRIQRGPGRAFVTIGK (SEQ ID
NO:4), DRVIEVVQGAYRAIRHIPRRIRQGLERRIQRGPGRAFVTIGK (SEQ ID
NO:5), DRVIEVVQGAYRAIRRIQRGPGRAFVTIGK (SEQ ID NO:6),
AQGAYRAIRHIPRRIRRIQRGPGPRAFVTIGK (SEQ ID NO:7),
EQMHEDIISLWDQSLKPCVKRIHIGPGRAFYTTKN (SEQ ID NO:8),
KQIINMWQEVGKAMYAPPISGQIRRIHIGPGRAFYTTKN (SEQ ID NO:9),
RDNWRSELYKYKVVKIEPLGVAPTRIHIGPGRAFYTTKN (SEQ ID NO:10),
AVAEGTDRVIEVVQGAYRAIRHIPRRIRQGLERRIHIGPGRAFYTTKN (SEQ ID
NO:11), DRVIEVVQGAYRAIRHIPRRIRQGLERRIHIGPGRAFYTTKN (SEQ ID
NO:12), DRVIEVVQGAYRAIRRIHIGPGRAFYTTKN (SEQ ID NO:13) or
AQGAYRAIRHIPRRIRRIHIGPGRAFYTTKN (SEQ ID NO:14).

        Preferably, the CLUVAC includes the amino acid
sequence of SEQ ID NO:2, SEQ ID NO:9 or SEQ ID NO:12.
Antigenic peptides can be longer than the length specified
by the SEQ ID NOS. recited herein, i.e., the peptide can be
extended by adding one or more (e.g., 5, 10, 15, 20) amino
acids at the amino and/or carboxy termini of the peptide
with any given SEQ ID NO.

        The invention also encompasses methods for inducing
a protective mucosal CTL response in a subject in which the
soluble antigen is delivered IR. Preferably, the level of CTL
activity induced by IR immunization is at least 10% greater
than that induced by other routes of mucosal administration
(e.g., IN). More preferably, mucosal CTL activity induced by
IR immunization is at least 2-fold, more preferably, at least
5-fold, and most preferably, at least 10-fold greater than
that induced by other routes of mucosal immunization (e.g., IN
or IG).

        Subjects to which the methods of the invention are
applied are mammals, e.g., humans, non-human primates, cats or
mice.

        Also provided within the invention are immunogenic
compositions for inducing a protective mucosal CTL response in
a subject which are adapted for intrarectal administration.
The compositions comprise a purified soluble antigen
formulated for intrarectal delivery to the rectum, colon,

sigmoid colon, or distal colon. They may be formulated as a rectal enema, foam, suppository, or topical gel and generally comprise a base, carrier, or aabsorption-promoting agent adapted for intrarectal delivery.

In more detailed aspects, the immunogenic compositions of the invention may include a rectal emulsion or gel preparation, preferably wherein the soluble antigen is admixed with a homogenous emulsion or gel carrier, eg., a polyoxyethylene gel. Alternatively, the soluble antigen may be admixed with a rectally-compatible foam.

In other preferred aspects, the immunogenic compositions of the invention are formulated in a suppository. The suppository is comprised of a base or carrier specifically adapted for intrarectal delivery of the antigen. Preferred bases may be selected from a polyethyleneglycol, witepsol H15, witepsol W35, witepsol E85, propyleneglycol dicaprylate (Sefsol 228), Miglyol810, hydroxypropylcellulose-H (HPC), or carbopol-934P (CP). More preferably, the suppository comprises at least two base materials to optimize structural and delivery performance. In other aspects, the suppository includes a stabilizing agent to minimize intrarectal degradation of the soluble antigen.

To optimize intrarectal delivery, the immunogenic compositions of the invention also preferably include an absorption-promoting agent, for example a surfactant, mixed micelle, enamine, nitric oxide donor, sodium salicylate, glycerol ester of acetoacetic acid, clyclodextrin or beta-cyclodextrin derivative, or medium-chain fatty acid.

In yet additional aspects of the invention, immunogenic compositions are provided which include an adjuvant which enhances the CTL response. Suitable adjuvants are detoxified bacterial toxins, for example detoxified cholera toxin (CT), mutant cholera toxin (MCT), mutant- E. coli heat labile enterotoxin, and pertussis toxin. Preferably, the adjuvant is conjugated to a mucosal tissue or T cell binding agent, such as protein A, an antibody that binds a mucosal tissue- or T-cell-specific protein, or a ligand or peptide that binds a mucosal tissue- or T-cell-

specific protein. In more preferred aspects, the adjuvant is a recombinant cholera toxin (CT) having a B chain of CT substituted by protein A conjugated to a CT A chain, which exhibits reduced toxicity and enhances mucosal tissue binding

5 mediated by protein A. Alternatively the adjuvant may be conjugated to a protein or peptide that binds specifically to T cells, for example by binding CD4 or CD8 (eg., the HIV V3 loop or a T cell-binding peptide fragment of the HIV V3 loop).

Unless otherwise defined, all technical and

10 scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are

15 described below. In case of conflict, the present application, including definitions, will control. In addition, the materials, methods, and examples described herein are illustrative only and not intended to be limiting.

Other features and advantages of the invention,

20 e.g., prevention of viral or other infectious diseases, will be apparent from the following detailed description, from the drawings and from the claims.

Brief Description of the Drawings

25 — Fig. 1 is a series of line graphs showing the results of a CTL assay. IR *HIV-1* peptide immunization induced long lasting mucosal and systemic CTL activity.

Fig. 2 is a series of line graphs showing the results of a CTL assay. CT enhanced (but was not essential)

30 for induction of CTL by IR administration of antigenic peptide.

Fig. 3 is a series of line graphs showing the results of a CTL assay. CTL induced by IR immunization with an *HIV-1* gp160 peptide specifically lysed target cells

35 transfected with and expressing an *HIV-1* gp160 gene.

Fig. 4 is a series of line graphs showing the results of a CTL assay which indicated that IR induction of CTL was IL-12 dependent.

Fig. 5 is a bar graph showing that IR immunization protects against IR challenge with *HIV-1* gp160 expressing recombinant vaccinia virus.

Fig. 6A is a line graph showing the results of a CTL assay using SP cells obtained from animals six months after IR immunization with an antigenic peptide.

Fig. 6B is a line graph showing the results of a CTL assay using SP cells obtained from animals six months after intranasal (IN) immunization with antigenic peptide.

Fig. 7A is a bar graph showing the results of a CTL assay using PP as effector cells.

Fig. 7B is a bar graph showing the results of a CTL assay using SP cells as effector cells. PP and SP cells were obtained from animals thirty five days after mucosal (IR, IN, IG) or systemic (subcutaneous (SC)) immunization with an antigenic peptide.

Fig. 8A is a bar graph showing the results of a CTL assay using effector cells from wild-type BALB/c mice.

Fig. 8B is a bar graph showing the results of a CTL assay using effector cells from BALB/c mice which lack the ability to produce functional IFNγ. These experiments show the dependence of IR induction of CTL on IFNγ.

Fig. 9A is a line graph showing the results of a CTL assay using PP as effector cells.

— Fig. 9B is a line graph showing the results of a CTL assay using SP cells as effector cells. PP and SP cells were obtained from BALB/c mice thirty five days after IR immunization with either antigenic peptide, CT and IL-12 (composition A) or antigenic peptide and CT (without IL-12; composition B).

Figs. 10A and 10B demonstrate that IR immunization with PCLUS3-18IIIB or PCLUS3-18MN induces both Peyer's patch (panel A) and spleen (panel B) long-lasting immunity. However the levels of the induction of CTL are different. Killing of P18IIIB-I10 (closed squares) or P18MN-T10 peptide-pulsed targets (closed circles) is compared with killing of unpulsed targets (open squares or circles). In both panel A and panel B, SEM of triplicate were all <5% of the mean.

Fig. 11 demonstrates that protection induced by mucosal immunization with HIV-1 peptide vaccine is specific. On day 35, mice were challenged intrarectally with 2.5×10^7 plaque-forming units (pfu) of vaccinia virus expressing gp 160IIB (vPE16) or with 2.5×10^7 pfu of vaccinia virus expressing β-galactosidase (vSC8). Bars = SEM of five mice per group. The difference is significant at P< 0.01 by Student's test.

Fig. 12 demonstrates that protection induced by mucosal immunization with HIV-1 peptide vaccine is long-lasting. On day 35 or 6 months after the start of the immunization, mice were challenged intrarectally with 2.5×10^7 pfu of vaccinia virus expressing gp 160IIIB. Bars = SEM of five mice per group. The difference is significant at P<0.01 by Student's test.

Fig. 13 demonstrates that protection induced by mucosal immunization with HIV-peptide is dependent on CD8 positive T-cells. BALB/c mice were treated IP with 0.5 mg monoclonal anti-CD8 antibody (clone 2.43, NIH Frederick, MD) one day before and after each immunization and also two days before and three days after the challenge with vPE16. Mice were challenged intrarectally with 2×10^7 pfu of vPE16 vaccinia virus expressing gp 160IIIB.

Figs. 14A and 14B demonstrate that mucosal immunization with HIV-1 peptide induces mucosal CTL responses and stimulates protective immunity against intrarectal recombinant HIV-1 vaccinia challenge. Figure 14A depicts induction of the mucosal and systemic CTL responses by different routes of immunization with synthetic peptide HIV vaccine. Killing of peptide-pulsed targets (closed bars) is compared with killing of unpulsed targets (open bars) at an effector-to-target ratio of 50:1. Panel A depicts results or immunizing on day 35 (IR or SC--bar 1, no immunogen; bar 2, SC; bar 3, IR) BALB/c mice challenged intrarectally with 2.5×10^7 plaque-forming units (pfu) of vaccinia virus expressing gp 160IIIB.

Figs. 15A and 15B demonstrate enhancement of the mucosal (Fig. A) and systemic (Fig. B) CTL responses to HIV-1

peptide by the mucosal (not systemic) treatment with rmIL-12. BALB/c mice were treated by the IP route (right panels) or IR route (left panels) with 1μg of the rmIL-12 each day of the IR immunization with PCLUS3-18IIIB (50 μg/mice). On day 35 HIV-

5   specific Peyer's patch CTL (Fig. 15A) and spleen CTL (Fig. 15B) were studied.

Fig. 16 demonstrates that mucosal treatment with rmIL-12 in DOTAP along with HIV peptide vaccine enhances protection against mucosal challenge with vaccinia virus

10 expressing gp160IIIB (vPE16). Five mice per group were immunized IR on days 0, 7, 14 and 21 with no immunogen (bar 1), with 50 μg PCLUS3-18IIB alone (bar 2), or with peptide plus 1 μg rmIL-12 in DOTAP (bar 3), and challenged on day 35 intrarectally with 5 X 10^7 pfu of vaccinia virus expressing gp

15 160IIIB. Viral pfu in the ovaries were determined six days later.

Figs. 17A and 17B demonstrate that IL-12 cannot act directly in the induction of mucosal CD8+ CTL in the absence of IFNγ. IFNγ$^{-/-}$ mice (BALB/c background) were immunized IR

20 with the rmIL-12 (1 μg/mouse) + DOTAP together with peptide. On day 35 HIV-specific Peyer's patch CTL (Fig. 17A) and spleen CTL (Fig. 17B) were studied. Killing of P18IIIB-I10 pulsed targets by effector cells from immunized IFNγ$^{-/-}$ mice (closed squares) or conventional BALB/c mice (closed circles) is

25 compared with killing of unpulsed targets (open squares or circles).

Detailed Description
A.   INDUCTION OF MUCOSAL IMMUNITY

30 The present invention is based on the discovery that IR administration of a peptide, e.g., a synthetic multideterminant HIV-1 gp160 envelope glycoprotein peptide, to a mucosal surface can induce an antigen-specific, protective CTL response in the mucosal immune system, even in the absence

35 of a mucosal adjuvant.

The exemplary synthetic multideterminant peptides (CLUVAC) are composed of subregions containing epitopes that evoke some or all of (a) a helper T cell response, (b) a CTL

response and (c) a high titer of neutralizing antibodies in multiple hosts of a given species expressing a broad range of MHC haplotypes.   IR immunization with the *HIV-1* CLUVAC PCLUS3-18IIIB (SEQ ID NO:2), and the mucosal adjuvant CT induced

5   peptide-specific CTL in both the inductive (PP) and the effector (LP) sites of the mucosal immune system, as well as in systemic lymphoid tissue, i.e., SP.   In contrast, systemic immunization with peptide vaccine produced *HIV-1* peptide-specific CTL only in the SP.

10   IR immunization induced long-lasting protective immune responses.   For example, antigen-specific CTL were found in both mucosal and systemic sites 6 months after immunization.   IR immunization with the antigenic peptide elicited significantly stronger CTL responses than IN

15   immunization with the same peptide.   While IR administration with PCLUS3-18IIIB (SEQ ID NO:2) induced a significant response when administered alone, the response was enhanced by the inclusion of CT.   The CTL were CD8+ T lymphocytes restricted by MHC class I molecules, recognizing MHC class I

20   positive target cells either endogenously expressing *HIV-1* gp160 or pulsed with an appropriate gp160 peptide.   Induction of both mucosal and systemic CTL response by IR immunization was IL-12-dependent, as shown by inhibition of induction of CTL in mice treated i.p. with anti-IL-12 antibody,

25   Furthermore, inclusion of IL-12 in the composition of antigenic peptide and CT used for IR immunization resulted in enhanced mucosal and systemic CTL responses relative to the responses elicited by antigenic peptide and CT without IL-12. The dependence on IFNγ of mucosal and systemic CTL generation

30   following IR immunization was demonstrated by the absence of such responses in mice which lack the ability to produce functional IFNγ, e.g., as the result of a premature stop codon in the IFNγ-encoding gene.   The stop-codon mutation causes the gene to encode a truncated protein lacking the activity of

35   IFNγ.

       IR immunization with PCLUS3-18IIIB (SEQ ID NO:2) protected mice against an IR challenge with a recombinant vaccinia virus expressing *HIV-1* IIIB gp160.   Thus, an HIV-1

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peptide induced CTL responses in the mucosal and systemic immune systems after IR immunization and protected against mucosal challenge with virus expressing *HIV-1* gp160.

The immunization method of the invention is useful to induce a mucosal CTL response to any soluble antigen. Accordingly, the invention provides a new method for vaccine administration to elicit immunologic protection against viruses that enter through mucosal barriers, including *HIV-1*. The method can also be applied to achieving protection from infection by certain bacteria (e.g., L. *monocytogenes*) and protozoa (e.g., G. *lamblia*) that establish infection via mucosae. In addition, since mucosal immunization results in systemic generation of CTL activity, the protocol can also be useful in prevention of infection by microorganisms that enter through non-mucosal routes. The method is also useful for immunotherapy of infections that are mucosally or non-mucosally established. Finally, the methods can be utilized for immunotherapy of cancer, both in the region of and remote from a mucosal surface.

B. METHODS OF IMMUNIZATION

The invention features methods for protecting subjects from infection by intracellular microorganisms such as viruses as well as intracellular bacteria and protozoans. The methods involve induction of CTL responses specific for antigenic peptides derived from proteins encoded by genes of relevant microbes and expressed in association with MHC class I molecules on the surface of infected cells. This is achieved by delivery of an appropriate soluble antigen to a mucosal surface, e.g., rectal, vaginal, nasopharyngeal, gastric or tracheal mucosae. Relevant microorganisms include but are not restricted to those that enter their hosts via mucosal barriers, e.g. *HIV-1*, influenza virus, enteric viruses such as rotaviruses, hepatitis A virus, papilloma virus, feline immunodeficiency virus, feline leukemia virus, simian immunodeficiency virus, intracellular bacterial pathogens, e.g., L. *monocytogenes* and mycobacteria such as *M. tuberculosis* and *M. leprae*. Since the responses elicited by

mucosal immunization occur in the systemic as well as the
mucosal immune system, the methods can also be applied to
protection from infection by intracellular pathogens that
enter their hosts via non-mucosal (e.g., *HIV-1* in some
scenarios such as a "stick" by a contaminated syringe needle,
rabies virus and malarial protozoans) as well as mucosal
routes. In light of the above considerations, immunization
via mucosae can also be used for immunotherapy of
intracellular infections.

        Finally, the mucosal immunotherapy of the invention
can be applied to subjects with cancer, particularly, but
not limited to, those with solid tumors in the region of the
relevant mucosa, e.g., colonic, rectal, bladder, ovarian,
uterine, vaginal, prostatic, nasopharyngeal, lung or certain
melanoma tumors. Tumor immunity is substantially mediated by
CTL with specificity for tumor associated peptides (e.g.,
prostate specific antigen peptides in prostatic cancer,
carcinoembryonic antigen peptides in colon cancer, human
papilloma virus peptides in bladder cancer and MART1, gp100
and tyrosinase peptides in melanoma) (Rosenberg et al. (1994)
J.N.C.I. 76:1159; Kawakami et al. (1994) Proc. Natl. Acad.
Sci. USA. 91:3515) bound to MHC class I molecules on the
surface of tumor cells.

B.1   __   Antigenic Polypeptides

        A soluble antigen to be administered according to
the invention can be any soluble carbohydrate or peptide
antigen, e.g., one containing all or part of the amino acid
sequence of a peptide which is naturally expressed in
association with a MHC class I molecule on the surface of a
cell infected with relevant microbe or expressed on a tumor
cell. In the case of infected cells, the cell surface
expressed peptide is derived from a protein either encoded by
genes of the infectious agent or whose expression is induced
by the infectious agent. Thus, the soluble antigen can be a
full length, naturally occurring polypeptide, e.g., full
length *HIV-1* gp160 or gp120 or an antigenic fragment thereof.

Antigen-specific recognition by CTL involves interactions between components of the antigen-specific T cell receptor on the surface of the CTL and residues on both the antigenic peptide and the MHC class I molecule to which the

5   peptide is bound. Thus the soluble antigen can also be a fragment (i.e., a peptide) of the naturally occurring polypeptide that is either (a) itself capable of binding to MHC class I molecules of multiple haplotypes on the surface of antigen presenting cells (APC) and stimulating CD8+ T cell

10   responses in subjects expressing these MHC class I haplotypes or (b) which can be proteolytically processed by APC into fragments with these properties. Ways of establishing the ability of a candidate peptide to stimulate a CTL response in the context of multiple MHC class I haplotypes are well known

15   to one of ordinary skill in the art and are amply described in co-pending U.S. patent application 08/060,988 incorporated herein by reference in its entirety.

Antigenic peptides can be engineered to bind to MHC class II molecules of multiple haplotypes and will be

20   recognized by CD4+ helper T cell precursor cells of subjects expressing multiple MHC class II haplotypes. Ways of establishing the ability of a candidate peptide to stimulate a helper T cell response in the context of multiple MHC class II haplotypes are well known to one of ordinary skill in the art

25   and are amply described in co-pending U.S. patent application 08/060,988 incorporated herein by reference in its entirety.

In addition, antigenic peptides, can contain epitopes that elicit neutralizing antibodies, i.e., those that bind to the relevant microbe or a cell infected with the

30   microbe and neutralize or kill it. Ways of establishing these properties of a candidate peptide are well known to one of ordinary skill in the art and are amply described in copending U.S. patent application 08/060,988 incorporated herein by reference in its entirety.

35   Antigenic peptides can also elicit antibodies that induce antibody-dependent cellular cytolysis (ADCC) of cells infected by the appropriate microbe or tumor cells expressing at their surface the protein from which the antigenic peptide

was derived. ADCC is a protective mechanism by which specialized cells of the immune system (K cells) recognize the Fc portion of IgG antibody molecules bound to the surface of a target cell and lyse the relevant target cell. Sera, or other body fluids such as rectal lavages, from test subjects mucosally immunized with a candidate peptide can be tested for their ability to mediate ADCC by methods known to an ordinary artisan. A standard cell mediated lympholysis (CML) assay is used. Briefly, a source of lymphoid ADCC effector cells (e.g., peripheral blood mononuclear cells (PBMC) or SP cells) is incubated *in vitro* with target cells expressing the above described cell surface protein in the presence of various dilutions of test sera. Lysis of the target cells, which can be measured by the release of a detectable label (^{51}Cr, for example) from prelabeled target cells, is an indication of the presence of ADCC inducing antibodies in the test serum.

Peptides of the invention can be cluster peptide vaccine constructs (CLUVAC). A CLUVAC is a chimeric peptide containing a) a subregion with multiple overlapping helper T cell activating epitopes that can be presented by multiple MHC class II molecules (a cluster peptide), b) a subregion with a CTL activating epitope and c) a subregion that elicits the production of a neutralizing antibody. The peptide sequences containing these epitopes can be derived from different parts of a microbial or tumor associated polypeptide. Alternatively, the CTL inducing and antibody neutralizing epitopes can be located in one subregion of an antigen and the helper epitope(s) can be in a second subregion. CLUVAC and their design are extensively described in co-pending U.S. patent application 08/060,988 incorporated herein by reference in its entirety.

HIV-1 CLUVAC can include the following sequences:
EQMHEDIISLWDQSLKPCVKRIQRGPGRAFVTIGK (SEQ ID NO:1)
KQIINMWQEVGKAMYAPPISGQIRRIQRGPGRAFVTIGK (SEQ ID NO:2)
RDNWRSELYKYKVVKIEPLGVAPTRIQRGPGRAFVTIGK (SEQ ID NO:3)
AVAEGTDRVIEVVQGAYRAIRHIPRRIRQGLERRIQRGPGRAFVTIGK (SEQ ID NO:4)
DRVIEVVQGAYRAIRHIPRRIRQGLERRIQRGPGRAFVTIGK (SEQ ID NO:5)
DRVIEVVQGAYRAIRRIQRGPGRAFVTIGK (SEQ ID NO:6)

AQGAYRAIRHIPRRIRRIQRGPGRAFVTIGK (SEQ ID NO:7)
EQMHEDIISLWDQSLKPCVKRIHIGPGRAFYTTKN (SEQ ID NO:8)
KQIINMWQEVGKAMYAPPISGQIRRIHIGPGRAFYTTKN (SEQ ID NO:9)
RDNWRSELYKYKVVKIEPLGVAPTRIHIGPGRAFYTTKN (SEQ ID NO:10)

5   AVAEGTDRVIEVVQGAYRAIRHIPRRIRQGLERRIHIGPGRAFYTTKN (SEQ ID
NO:11)

DRVIEVVQGAYRAIRHIPRRIRQGLERRIHIGPGRAFYTTKN (SEQ ID NO:12)
DRVIEVVQGAYRAIRRIHIGPGRAFYTTKN (SEQ ID NO:13)
AQGAYRAIRHIPRRIRRIHIGPGRAFYTTKN (SEQ ID NO:14)

10   It is possible, for example, to link a particular
cluster peptide to any peptide containing a CTL and/or
neutralizing antibody epitope. Examples of cluster peptides
include: cluster peptide 1 whose amino acid sequence
(EQMHEDIISLWDQSLKPCVK) (SEQ ID NO:17) is the first 20 amino

15   acids of the *HIV-1* CLUVAC with SEQ ID NO:1; cluster peptide 3
whose amino acid sequence (KQIINMWQEVGKAMYAPPISGQIR) (SEQ ID
NO:18) is the first 24 amino acids of the *HIV-1* CLUVAC with
SEQ ID NO: 2; cluster peptide 4 whose amino acid sequence
(RDNWRSELYKYKVVKIEPLGVAPT) (SEQ ID NO:19) is the first 24

20   amino acids of the *HIV-1* CLUVAC with SEQ ID NO:3; and cluster
peptide 6 whose amino acid sequence
(AVAEGTDRVIEVVQGAYRAIRHIPRRIRQGLER) (SEQ ID NO:20) is the
first 33 amino acids of the *HIV-1* CLUVAC with SEQ ID NO:4.
             Of particular interest are peptides containing the

25   amino acid sequences of SEQ ID NO:2, SEQ ID NO:9 and SEQ ID
NO:12. Mucosal responses to the first (PCLUS3-18IIIB) are
extensively characterized in the Examples presented *infra*.
PCLUS3-18MN (SEQ ID NO:9) and PCLUS6.1-18MN (SEQ ID NO:12)
have been tested in human clinical trials. The CTL epitope

30   within PCLUS3-18IIIB (SEQ ID NO:2) is the amino acid sequence
RIQRGPGRAFVTIGK (SEQ ID NO:15).
             In some instances, mucosal adjuvants are co-
administered at the mucosal tissue site with the soluble
antigens. Such adjuvants include, but are not limited to,

35   detoxified bacterial toxins, for example detoxified cholera
toxin (CT), E. coli heat labile toxin (LT), mutant CT (MCT)
(Yamamoto et al. J. Exp. Med. 185:1203 (1997); Yamamoto et
al. Proc. Natl. Acad. Sci. USA 58:5267 (1997); Douce et al.

Infect. Immunity 65:2821 (1997)), mutant E. coli heat labile toxin (MLT) (Di Tommaso et al. *Infect. Immunity 64*:974 (1996); Partidos et al. *Immunology 89*:83 (1996), and pertussis toxin. MCT and MLT contain point mutations that substantially ablate toxicity without substantially compromising adjuvant activity relative to that of the parent molecules. In other preferred embodiments, the adjuvant is modified for increased binding to mucosal tissues and/or T-cells. Thus, in one aspect of the invention, CT or other bacterial toxins are conjugated to a mucosal tissue binding agent, such as protein A, an antibody that binds a mucosal tissue- or T-cell-specific protein (eg., a receptor), or a ligand or peptide that binds a mucosal tissue- or T-cell-specific protein (eg., CD4 or CD8). For example, the B chain of CT may be substituted by protein A, conjugated to the CT A chain, to eliminate toxicity and enhance mucosal tissue binding mediated by protein A. Alternatively, the HIV V3 loop which binds CD4 on T cells may be conjugated to the adjuvant to enhance delivery to T cells.

The CTL augmenting cytokines, eg., IL-12 and IFNγ, are, in some instances, either administered systemically or, preferably, coadministered at the mucosal tissue site with the soluble antigen.

Variants of disclosed antigenic peptides can contain different amino acids (preferably conservative changes) from the parental molecules but retain the biological activity of the parental molecules, e.g., the ability to induce specific CTL responses subsequent to mucosal immunization. Such variants can be synthesized by standard means, and are readily tested in assays known to those in the art. Antigenic polypeptides are typically longer than the length of the nominal SEQ ID NOS. recited herein, i.e., the polypeptide can be extended by adding amino acids to the amino or carboxy termini of the peptide defined by any given SEQ ID NO.

Peptides and polypeptides of the invention will also include those described above but modified for *in vivo* use by:

(a)   chemical or recombinant DNA methods to include mammalian signal peptides (Lin et al., J. Biol. Chem. 270:14255 (1995)) or the bacterial peptide, penetratin (Joliot et al., Proc. Natl. Acad. Sci. USA 88:1864 (1991)), that will

5   serve to direct the peptide across cell and cytoplasmic membranes and/or traffic it to the endoplasmic reticulum (ER) of antigen presenting cells (APC), e.g., dendritic cells which are potent CTL inducers;

(b)   addition of a biotin residue which serves to

10   direct the polypeptides or peptide across cell membranes by virtue of its ability to bind specifically to a translocator present on the surface of cells (Chen et al., Analytical Biochem. 227:168 (1995));

(c)   addition at either or both the amino- and

15   carboxy-terminal ends, of a blocking agent in order to facilitate survival of the relevant polypeptide or peptide *in vivo*. This can be useful in those situations in which the termini tend to be degraded ("nibbled") by proteases prior to cellular or ER uptake. Such blocking agents can include,

20   without limitation, additional related or unrelated peptide sequences that can be attached to the amino and/or carboxy terminal residues of the polypeptide or peptide to be administered. This can be done either chemically during the synthesis of the peptide or by recombinant DNA technology (see

25   Section 3.3 *infra*). Alternatively, blocking agents such as pyroglutamic acid or other molecules known to those of average skill in the art can be attached to the amino and/or carboxy terminal residues, or the amino group at the amino terminus or carboxyl group at the carboxy terminus replaced with a

30   different moiety. Likewise, the polypeptides or peptides can be covalently or noncovalently coupled to pharmaceutically acceptable "carrier" proteins prior to administration.

Also of interest are peptidomimetic compounds based upon the amino acid sequence of the peptides of the invention.

35   Peptidomimetic compounds are synthetic compounds having a three-dimensional structure (i.e. a "peptide motif") based upon the three-dimensional structure of a selected peptide. The peptide motif provides the peptidomimetic compound with

the activity of binding to MHC molecules of multiple
haplotypes and activating CD8⁺ and CD4⁺ T cells from subjects
expressing such MHC molecules that is the same or greater than
the activity of the peptide from which the peptidomimetic was

5   derived. Peptidomimetic compounds can have additional
characteristics that enhance their therapeutic application
such as increased cell permeability, greater affinity and/or
avidity and prolonged biological half-life. The
peptidomimetics of the invention typically have a backbone

10   that is partially or completely non-peptide, but with side
groups identical to the side groups of the amino acid residues
that occur in the peptide on which the peptidomimetic is
based. Several types of chemical bonds, e.g. ester,
thioester, thioamide, retroamide, reduced carbonyl,

15   dimethylene and ketomethylene bonds, are known in the art to
be generally useful substitutes for peptide bonds in the
construction of protease-resistant peptidomimetics.

B.2 *In vivo* methods to deliver soluble antigens to mucosae of
20   a subject

            In methods of the invention that induce mucosal CTL
responses, a soluble antigen is delivered to antigen
presenting cells of the inductive mucosal immune system (e.g.,
PP of the intestine). Delivery involves administering to a

25   subject either the soluble antigen itself, e.g., an antigenic
peptide, an expression vector encoding the soluble antigen, or
cells transfected or transduced with the vector.

B.2.1 Administration of soluble antigen
30   Soluble antigens can be delivered to the mucosal
immune system of a mammal using techniques substantially the
same as those described *infra* for delivery to human subjects.
Examples of appropriate mammals include but are not restricted
to humans, non-human primates, horses, cattle, sheep, dogs,

35   cats, mice, rats, guinea pigs, hamsters, rabbits and goats.
            A soluble antigen of the invention can be delivered
to the mucosal immune system of a human in its unmodified
state, dissolved in an appropriate physiological solution,

- 20 -

e.g., physiological saline. Alternatively, it can be modified as detailed in Section B.1 in order to facilitate transport across cell and/or intracellular membranes and to prevent extracellular or intracellular degradation. Its transport

5   across biological membranes can also be enhanced by delivering it encapsulated in liposomes using known methods (Gabizon et al., _Cancer Res._ 50:6371 (1990); Ranade, _J. Clin. Pharmacol._ 29:685 (1989)) or an appropriate biodegradable polymeric microparticle (also referred to as a "microsphere",

10 "nanosphere", "nanoparticle, or "microcapsule"). Naturally, it is desirable that the soluble antigens be selectively targeted to the mucosae. This can be achieved by contacting the soluble antigens directly with the relevant mucosal surface, e.g., by IR, IVG, IN, intrapharyngeal (IPG) or IT

15 infusion or implantation. CTL activity (systemic or mucosal) induced by IR immunization can be two-fold, preferably five-fold, more preferably twenty-fold, even more preferably fifty-fold and most preferably two hundred-fold greater than that induced by IN immunization.

20   Soluble antigens of the invention can be delivered in liposomes into which have been incorporated ligands for receptors on relevant cells (e.g., dendritic cell or macrophage APC) or antibodies to cell-surface markers expressed by these cells. Thus, for example, an antibody

25 specific for a dendritic cell surface marker can direct liposomes containing both the anti-dendritic cell antibody and the relevant soluble antigen to dendritic cells.

          The soluble antigens can be administered mucosally either alone or together with a mucosal adjuvant. Suitable

30 adjuvants include, but are not restricted to, CT, MCT and MLT. IL-12 and/or IFNγ can also be administered to the subject. Thus, a soluble antigen can be administered with an adjuvant, with IL-12 (in the absence of an adjuvant), with IFNγ (in the absence of an adjuvant), with both IL-12 and IFNγ (in the

35 absence of an adjuvant), with an adjuvant and IL-12, with an adjuvant and IFNγ, or with an adjuvant and both IL-12 and IFNγ. The IL-12 and IFNγ can be administered systemically or co-administered mucosally with the peptide. When the soluble

antigen is administered encapsulated in liposomes or
microparticles, the IL-12 and/or IFNγ can be co-incorporated
into the same liposomes/microparticles or incorporated into
separate liposomes/microparticles. Alternatively, the
5   cytokines can be administered in a free, soluble form.
Examples of other cytokines that can be used, singly or in
combination, are granulocyte-macrophage colony-stimulating
factor (GM-CSF), interleukin-2 (IL-2), interleukin-7 (IL-7),
or tumor necrosis factor a (TNFa). These and other
10   immunopotentiating cytokines are administered as discussed
above for IL-12 and IFNγ. Administration can be single or
multiple (two, three, four, five, six, eight or twelve
administrations, for example). Where multiple, they can be
spaced from one day to one year apart. When using peptides
15   without a helper T-cell epitope, it can be necessary to carry
out such multiple administrations.

        In preferred aspects of the invention formulations
of soluble antigen are prepared for intrarectal administration
(i.e., delivery to the rectum, colon, sigmoid colon, or distal
20   colon), eg., by formulating the soluble antigen in a rectal
enema, foam, suppository, or topical gel. These rectal
delivery formulations are adapted for improved delivery,
stability, and/or absorption in the rectum, eg., by combining
the soluble antigen with one or more known intrarectal
25   delivery base or carrier materials, intrarectal absorption-
promoting materials, and/or stabilizers.

        Preferred base formulations for rectal delivery of
soluble antigen within the methods of the invention include
hydrophilic and hydrophobic vehicles or carriers such as those
30   commonly used in formulating suppositories and rectal emulsion
or gel preparations. Thus, emulsion vehicles are used which
incorporate soluble antigen in an oil/water emulsion suitable
for intrarectal administration. Alternatively, gel
formulations are provided which incorporate the soluble
35   antigen in a homogenous gel carrier, for example a
polyoxyethylene gel such as polyoxyethylene(20)cetylether(BC-
20TX). When the antigen is formulated in a rectally
compatible foam, a non-CFC propellant foam is preferred.

Preferred carriers or delivery vehicles for use within the invention are conventional suppository base materials that are adapted for intrarectal use, such as polyethyleneglycols. Exemplary polyethyleneglycols known and

5   available in art include PEG 400, PEG 1500, PEG 2000, PEG 4000 or PEG 6000. Preferred bases in this context include witepsol H15, witepsol W35, witepsol E85. These are selected for their desired lipophilic and/or hydrophilic properties, and are often selected to form a multiple layer suppository with both

10   lipophilic and hydrophilic base layers. Preferred lipophilic base materials are macrogols of low molecular weight. Particular examples of suitable lipophilic bases include propyleneglycol dicaprylate (Sefsol 228) and Miglyol810. A preferred hydrophilic base is PEG, eg., PEG 400. In one

15   formulation useful within the invention, hydroxypropylcellulose-H (HPC) and/or carbopol-934P (CP) are used as bases for an inner suppository layer, and a witepsol base is used for the outer layer.

Crystalline cellulose or other common stabilizing

20   agents can be added in selected amounts (eg., 30-60% by weight) to the base to promote sustained release.

Selection of base materials, stabilizers, and absorption-promoting agents for formulating intrarectal delivery compositions, particularly suppositories, is

25   determined according to conventional methods, eg., based on melting and drop rates, breaking hardness, disintegration and special breaking times, spreading properties, and diffusion rates. Preferred values of these various properties are known and can be readily determined to adjust antigen formulations

30   to be suitable for intrarectal use. In particular, appropriate values for formulating a time-release suppository having appropriate structural and chemical properties for rectal delivery are known or readily ascertained. In this context, conventional additives, eg., softeners such as

35   neutral oils, Estasan, etc. may be included to optimize consistency, rate of delivery and other characteristics of the formulation.

In formulating mucosal delivery compositions, it is
also desired to include absorption-promoting agents to enhance
delivery of the soluble antigen and, optionally the CTL-
stimulatory cytokine, to the mucosal surface. A variety of
5   absorption promoting agents (i.e., agents which enhance
release or diffusion of the antigen and/or CTL-stimulatory
cytokine from the delivery vehicle or base, or enhance
delivery of the antigen and/or CTL-stimulatory cytokine to the
mucosal tissue or T-cells, for example by enhancing membrane
10   penetration) are known in the art and are useful in mucosal
delivery formulations of the invention, eg., for inclusion in
intrarectal delivery formulations, particularly suppositories.
These include, but are not limited to, surfactants (eg., tween
80), mixed micelles, enamines, nitric oxide donors (eg., S-
15   nitroso-N-acetyl-DL-penicillamine, NOR1, NOR4--which are
preferably co-administered with an NO scavenger such as
carboxy-PITO or doclofenac sodium), sodium salicylate,
glycerol esters of acetoacetic acid (eg., glyceryl-1,3-
diacetoacetate or 1,2-isopropylideneglycerine-3-acetoacetate)
20   and other release-diffusion/absorption-promoting agents
adapted for mucosal delivery.

        Absorption-promoting agents useful within the
invention include a variety of compounds specifically adapted
for intrarectal use. In this context, the rate and extent of
25   rectal drug absorption are often lower than with oral
absorption, possibly an inherent factor owing to the
relatively small surface area available for drug uptake. In
addition, the composition of rectal formulations (solid vs
liquid, nature of the suppository base) is an important factor
30   in the absorption process. This relationship between
formulation and drug uptake has been clearly demonstrated.
Thus, coadministration of absorption-promoting agents (eg.,
surfactants, sodium salicylate, enamines) represents a key
approach towards optimizing rectal drug absorption.

35   Rectal drug delivery in a site- and rate-controlled
manner using suppositories, enemas, osmotic pumps, or hydrogel
formulations provides a range of options for manipulating
mucosal delivery, eg., controlling concentrations, time-

release, and immunogen-drug effects. Absorption from aqueous
and alcoholic solutions may occur very rapidly, but absorption
from suppositories is generally slower and very much dependent
on the nature of the suppository base, the use of surfactants
5   or other additives, particle size of the active ingredients,
etc.

Accordingly, preferred formulations for
administering soluble antigens and CTL-stimulatory cytokines
within the methods of the invention are designed to optimize
10   mucosal delivery. These agents may thus include
clyclodextrins and beta-cyclodextrin derivatives (eg., 2-
hydroxypropyl-beta-cyclodextrin and heptakis(2,6-di-O-methyl-
beta-cyclodextrin). These compounds, preferably conjugated
with one or more of the active ingredients and formulated in
15   an oleaginous base, are well documented to enhance
bioavailability in intrarectal formulations. Other
absorption-enhancing agents adapted for intrarectal delivery
include medium-chain fatty acids, including mono- and
diglycerides (eg., sodium caprate--extracts of coconut oil,
20   Capmul), and triglycerides (eg., amylodextrin, Estaram 299,
Miglyol 810).

It is well known in the medical arts that dosages
for any one human subject depend on many factors, as well as
the particular compound to be administered, the time and route
25   of administration and other drugs being administered
concurrently. Dosages for the soluble antigens of the
invention will vary, but can be approximately 0.01 mg to 100
mg per administration. Dosages for the mucosal adjuvants will
be approximately 0.001 mg to 100 mg per administration.
30   Dosages for IL-12 will be approximately 25 μg/kg to 500 μg/kg
and for IFNγ will be 300 KU to 30,000 KU per administration--
comparable dosages will be used for other cytokines. For
example, 3,000 KU of IFNγ can be administered to a human
patient once per week. Methods of determining optimal doses
35   are well known to pharmacologists and physicians of ordinary
skill. Routes will be, as recited *supra*, mucosal, e.g., IR,
IG, IVG, IN, IPG or IT.

B.2.2 Administration of soluble antigens utilizing expression
vectors encoding the soluble antigens.

An expression vector is composed of or contains a
nucleic acid in which a polynucleotide sequence encoding a
5   peptide or polypeptide of the invention is operatively
linked to a promoter or enhancer-promoter combination. A
promoter is a trancriptional regulatory element composed of a
region of a DNA molecule typically within 100 nucleotide pairs
upstream of the point at which transcription starts. Another
10   transcriptional regulatory element is an enhancer. An
enhancer provides specificity in terms of time, location and
expression level. Unlike a promoter, an enhancer can function
when located at variable distances from the transcription
site, provided a promoter is present. An enhancer can also be
15   located downstream of the transcription initiation site. A
coding sequence of an expression vector is operatively linked
to a transcription terminating region. To bring a coding
sequence under control of a promoter, it is necessary to
position the translation initiation site of the translational
20   reading frame of the peptide or polypeptide between one and
about fifty nucleotides downstream (3') of the promoter.
Examples of particular promoters are provided *infra*.
Expression vectors and methods for their construction are
known to those familiar with the art.
25   —   Suitable vectors include plasmids, and viral
vectors such as herpes viruses, retroviruses, vaccinia
viruses, attenuated vaccinia viruses, canary pox viruses,
adenoviruses and adeno-associated viruses, among others.
The application of antigen-encoding genes to the
30   mucosal induction of CTL in humans can utilize either *in vivo*
or *ex vivo* based approaches.
The *ex vivo* method includes the steps of harvesting
cells (e.g., dendritic cells) from a subject, culturing the
cells, transducing them with an expression vector, and
35   maintaining the cells under conditions suitable for expression
of the soluble antigen. These methods are well known in the
art of molecular biology. The transduction step is
accomplished by any standard means used for *ex vivo* gene

therapy, including calcium phosphate, lipofection, electroporation, viral infection, and biolistic gene transfer. Cells that have been successfully transduced are then selected, for example, for expression of a drug resistance gene. The cells can then be lethally irradiated (if desired) and injected or implanted into the subject. IL-12 and IFNγ can be administered either systemically or to the relevant mucosal surface as discussed above (Section B.2.1).

The *in vivo* approach requires delivery of a genetic construct directly into the mucosa of a subject, preferably targeting it to the cells or tissue of interest (e.g., dendritic cells in PP). This can be achieved by administering it directly to the relevant mucosa (e.g., IR in the case of PP). Tissue specific targeting can also be achieved by the use of a molecular conjugate composed of a plasmid or other vector attached to poly-L-lysine by electrostatic or covalent forces. Poly-L-lysine binds to a ligand that can bind to a receptor on target cells (Cristiano et al. J. Mol. Med 73:479 (1995)). Similarly, cell specific antibodies of the type described *supra* in Section B.2.1 can be bound to vectors and thereby target them to the relevant cells of the mucosal immune system. A promoter inducing relatively tissue or cell-specific expression can be used to achieve a further level of targeting. Appropriate tissue-specific promoters include, for example, the inducible IL-2 (Thompson et al., Mol. Cell. Biol. 12: 1043 (1992)), IL-4 (Todd et al., J. Exp. Med. 177:1663 (1993)) and IFNγ (Penix et al., J. Exp. Med. 178:483 (1993)) T-cell targeting promoters. These promoters would allow production of the soluble antigens in lymphoid tissue, including mucosal lymphoid tissue, e.g., PP. Naturally, an ideal promoter would be a dendritic cell specific promoter.

Vectors can also be delivered by incorporation into liposomes or other delivery vehicles either alone or co-incorporated with cell specific antibodies, as described *supra* in Section B.2.1.

DNA or transfected cells can be administered in a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are biologically compatible vehicles which

are suitable for administration to a human, e.g.,
physiological saline. A therapeutically effective amount is
an amount of the DNA of the invention which is capable of
producing a medically desirable result in a treated animal.

5   As is well known in the medical arts, the dosage for any one
patient depends upon many factors, including the patient's
size, body surface area, age, the particular compound to be
administered, sex, time and route of administration, general
health, and other drugs being administered concurrently.

10   Dosages will vary, but a preferred dosage for administration
of DNA is from approximately 10^6 to 10^{12} copies of the DNA
molecule. This dose can be repeatedly administered, as
needed. Routes of administration will be the mucosal routes
recited for soluble antigens *supra* in Section B.2.1. The

15   mucosal adjuvants, IL-12 and IFNγ can also be administered in
the same combinations, by the same routes and at the same
dosages recited in Section B.2.1.

B.3   Sources of peptides and polypeptides

20   Peptides and polypeptides used in the methods of
the invention can be obtained by a variety of means. Smaller
peptides (less than 100 amino acids long) can be conveniently
synthesized by standard chemical methods familiar to those
skilled in the art (e.g, see Creighton, Proteins: Structures

25   and Molecular Principles, W.H. Freeman and Co., N.Y. (1983)).
Larger peptides (longer than 100 amino acids) can be produced
by a number of methods including recombinant DNA technology
(see *infra*). Some polypeptides (e.g., *HIV-1*, gp160, CT, LT,
IL-12, or IFNγ) can be purchased from commercial sources.

30   Polypeptides such as *HIV-1*, gp160, CT, IL-12, or
IFNγ can be purified from biological sources by methods well-
known to those skilled in the art (Protein Purification,
Principles and Practice, second edition (1987) Scopes,
Springer Verlag, N.Y.). They can also be produced in their

35   naturally occurring, truncated, chimeric (as in the CLUVAC,
for example), or fusion protein forms by recombinant DNA
technology using techniques well known in the art. These
methods include, for example, *in vitro* recombinant DNA

techniques, synthetic techniques, and *in vivo* genetic
recombination. See, for example, the techniques described in
Sambrook et al. (1989) Molecular Cloning, A Laboratory Manual,
Cold Spring Harbor Press, N.Y.,; and Ausubel et al., eds.
5   (1989), Current Protocols in Molecular Biology, Green
Publishing Associates, Inc., and John Wiley & Sons, Inc.,
N.Y.. Alternatively, RNA encoding the proteins can be
chemically synthesized. See, for example, the techniques
described in Oligonucleotide Synthesis, (1984) Gait, M.J. ed.,
10   IRL Press, Oxford, which is incorporated by reference herein
in its entirety.

A variety of host-expression vector systems can be
utilized to express the nucleotide sequences. Where the
peptide or polypeptide is soluble, it can be recovered from:
15   (a) the culture, i.e., from the host cell in cases where the
peptide or polypeptide is not secreted; or (b) from the
culture medium in cases where the peptide or polypeptide is
secreted by the cells, The expression systems also encompass
engineered host cells that express the polypeptide *in situ*,
20   i.e., anchored in the cell membrane. Purification or
enrichment of the polypeptide from such an expression system
can be accomplished using appropriate detergents and lipid
micelles and methods well known to those skilled in the art.
Alternatively, such engineered host cells themselves can be
25   used in situations where it is important not only to retain
the structural and functional characteristics of the protein,
but also to assess biological activity.

The expression systems that can be used for
purposes of the invention include but are not limited to
30   microorganisms such as bacteria (for example, *E. coli* and *B.
subtilis*) transformed with recombinant bacteriophage DNA,
plasmid DNA or cosmid DNA expression vectors containing the
nucleotide sequences; yeast transformed with recombinant yeast
expression vectors; insect cells infected with recombinant
35   viral expression vectors (baculovirus); plant cell systems
infected with recombinant viral expression vectors (e.g.,
cauliflower mosaic virus, CAMV; tobacco mosaic virus, TMV) or
transformed with recombinant plasmid expression vectors; or

mammalian cells (e.g., COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses.

In bacterial systems, a number of expression vectors can be advantageously selected depending upon the use intended for the gene product being expressed. For example, when a large quantity of such a protein is to be produced, e.g., for in vivo immunization, vectors which direct the expression of high levels of fusion protein products that are readily purified can be desirable. Such vectors include, but are not limited to, the E. coli expression vector pUR278, (Ruther et al., EMBO J. 2:1791 (1983)), in which the coding sequence can be ligated individually into the vector in frame with the lacZ coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, Nucleic Acids Res. 13:3101 (1985); Van Heeke & Schuster, J. Biol. Chem. 264:5503 (1989)); and the like. pGEX vectors can also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The PGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In mammalian host cells, a number of viral-based expression systems can be utilized. In cases where an adenovirus is used as an expression vector, the nucleotide sequence of interest can be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene can then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the gene product in infected hosts (e.g., See Logan and Shenk, Proc. Natl. Acad. Sci. USA 81:3655 (1984)). Specific

initiation signals can also be required for efficient translation of inserted nucleotide sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire gene or cDNA, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the coding sequence is inserted, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression can be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (Bittner et al., _Methods in Enzymol._ _153_:516 (1987)).

In addition, a host cell strain can be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. Mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, and WI38.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the sequences described above can be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells can be allowed to grow for 1-2 days in

an enriched medium, and then are switched to a selective
medium. The selectable marker in the recombinant plasmid
confers resistance to the selection and allows cells to stably
integrate the plasmid into their chromosomes and grow to form
5   foci which in turn can be cloned and expanded into cell lines.
This method can advantageously be used to engineer cell lines
which express the gene product. Such engineered cell lines
can be particularly useful in screening and evaluation of
compounds that affect the endogenous activity of the gene
10   product.

A fusion protein can be readily purified by
utilizing an antibody or a ligand that specifically binds to
the fusion protein being expressed. For example, a system
described by Janknecht et al., <u>Proc. Natl. Acad. Sci. USA</u>
15   <u>88</u>:8972 (1991) allows for the ready purification of non-
denatured fusion proteins expressed in human cell lines. In
this system, the gene of interest is subcloned into a vaccinia
recombination plasmid such that the gene's open reading frame
is translationally fused to an amino-terminal tag consisting
20   of six histidine residues. Extracts from cells infected with
recombinant vaccinia virus are loaded onto Ni_{2+} nitriloacetic
acid-agarose columns and histidine-tagged proteins are
selectively eluted with imidazole-containing buffers. If
desired, the histidine tag can be selectively cleaved with an
25   appropriate enzyme.

The following examples are meant to illustrate the
invention and not to limit it.

EXAMPLES

30   <u>Materials and Methods</u>
<u>Human IR immunization</u> Soluble antigens of the invention are
dissolved in a pharmaceutically acceptable carrier, e.g.,
physiological saline and administered with and without
adjuvant and with or without cytokines, e.g., IL-12 and/or
35   IFNγ. The latter can be administered systemically or together
with the antigen. Dosages of soluble antigen are
approximately 0.001 mg to 100 mg per administration.
Administration can be single, or multiple. In the case of

multiple immunizations, there can, for example, be four weekly
administrations, followed (if desired) by a booster
administration several months (e.g., two, three, four, six,
eight or twelve) or several years (e.g., two, three, four,
5   five, ten, twenty or thirty) thereafter. Administration of
the antigen (with and without adjuvant and with and without
cytokine) can be by IR insertion of a suppository into which
the immunizing composition has been incorporated, by enema or
by flexible sigmoidoscope.
10

CTL activation cultures   At various time points after
immunization (different for each experiment), CTL activity was
assessed. BALB/c mice were sacrificed and the intestines and
SP were surgically removed. Single cell suspensions were
15   prepared from the organs by methods familiar to those of
average skill in the art. In the case of PP and LP, the PP
were first dissected from the outer surface of the intestine.
The rest of the intestine was cut into small fragments 1 to 3
mm square which were suspended in phosphate buffered saline
20   (PBS). The tissue fragment suspension was stirred for 20
minutes at room temperature and shaken under the same
conditions to liberate the intraepithelial lymphocytes (IEL)
from the tissue. The suspended IEL were discarded and tissue
fragments were treated with collagenase type VIII (Sigma)
25   (300U/ml) dissolved in PBS for 1 hour at room temperature to
release the LP cells. PP cells were extracted from the
dissected PP by the same collagenase treatment. Cells (PP or
LP) were then placed on a discontinuous gradient containing
75% and 40% of Percoll, followed by centrifugation at 2,000
30   r.p.m. for 20 minutes. Lymphocytes were harvested from the
75%/40% interface and washed two times. Immune cells from SP,
PP, LP were cultured with $1\mu M$ P18IIIB-I10 peptide at 5×10^6
per/milliliter in 24-well culture plates in complete T cell
medium (CTM): RPMI 1640 containing 10% fetal bovine serum, 2mM
35   L-glutamine, penicillin (100U/ml), streptomycin ($100\mu g/ml$),
and 5×10^{-5} M 2-mercaptoethanol. P18IIIB-I10 is a peptide
containing the minimal essential CTL epitope of P18IIIB (SEQ
ID NO:15) and has the sequence: RGPGRAFVTI (SEQ ID NO:16).

Three days later, 10% supernatant from concanavalin A activated spleen cells was added as a source of interleukin-2 (IL-2). After 7 days of culture, LP lymphocytes (LPL) were stimulated in a second 7 day culture with 1µM P18IIIB-I10 peptide (SEQ ID NO:16) together with 4 X 10⁶ of 3300-rad irradiated syngeneic SP cells. Immune SP and PP cells were similarly stimulated in vitro for two 7-day culture periods before assay. Cytolytic activity of CTL was measured by a 4-hour assay with ⁵¹Cr labeled P815 targets using a method familiar to those of ordinary skill in the art. For testing the peptide specificity of CTL, ⁵¹Cr-labeled P815 targets were pulsed for 2 hours with peptide at the beginning of the assay. The percent specific ⁵¹Cr release was calculated as 100X (experimental release-spontaneous release)/(maximum release-spontaneous release). Maximum release was determined from supernatants of cells that were lysed by addition of 5% Triton-X 100. As a control, spontaneous release was determined from target cells incubated without added effector cells. Standard errors of the mean of triplicate cultures were all <5% of the mean.

Viral plaque assay Six days after IR challenge with recombinant vaccinia virus expressing HIV-1 IIIB gp160 (vPE16), mice were sacrificed. Their ovaries were removed, dissociated into single cell suspensions and assayed for vPE16 titer by plating serial 10-fold dilutions on a plate of BSC-2 indicator cells, staining with crystal violet and counting plaques at each dilution. The minimal detectable level of virus was 70 plaque forming units (pfu).

Example 1. Comparison of mucosal and systemic CTL responses after mucosal and systemic immunization

Mice were immunized IR with 4 doses (on days 0, 7, 14 and 21) of the HIV-1 CLUVAC PCLUS3-18IIIB (SEQ ID NO:2) (50µg/mouse). 5 weeks to 6 months after the first dose, antigen-specific T cells were isolated from PP, LP and SP and tested for the presence of HIV-1 P18IIIB peptide specific CTL (Fig. 1), as described above. Closed squares show killing of

P18IIIB-I10 (SEQ ID NO:16) -pulsed targets and open diamonds
show killing on unpulsed targets. IR immunization induced
long-lasting protective immune responses: antigen-specific CTL
were detected in mucosal inductive (PP) and effector (LP)
5   sites and in a systemic site (SP) at least 6 months after
immunization. In contrast, systemic immunization (s.c. in
incomplete Freund's adjuvant) induced CTL in the spleen but
not in the mucosal immune system (i.e., PP and LP).

10   **Example 2. Comparison of CTL responses with and
                     without a mucosal adjuvant**
              BALB/c mice were immunized IR with 4 doses of the
synthetic *HIV-1* CLUVAC PCLUS3-18IIIB (SEQ ID NO:2) (50µg/mouse
per immunization) alone, i.e., in the absence of adjuvant or
15   cytokine on days 0, 7, 14 and 21. In parallel, another group
of mice was immunized IR with PCLUS3-P18IIIB (SEQ ID NO:2)
HIV-1 peptide in combination with CT (1µg/mouse). On day 35
antigen-specific T cells were isolated from PP, LP and SP.
Immune cells from SP, PP, or LP were cultured and tested for
20   antigen specific CTL (Fig. 2), as described above. Closed
squares show killing of P18IIIB-I10 (SEQ ID NO:16) pulsed
targets, and open diamonds show killing of unpulsed targets.
IR administration of peptide alone induced a significant
response. The response was enhanced by the co-administration
25   of CT. _

       **Example 3. CTL induced by mucosal immunization lyse targets
              expressing *HIV-1* gp160 envelope protein**
              Mice were immunized IR with 4 doses (on days 0, 7,
30   14 and 21) of the *HIV-1* CLUVAC PCLUS3-18IIIB (SEQ ID NO:2)
(5µg/mouse per immunization) in the presence of CT
(1µg/mouse).. on day 35, antigen-specific T cells were isolated
from PP, LP and SP. Immune cells from SP, PP, or LP were
cultured as described above. Cytolytic activity of CTL was
35   measured using a standard ^{51}Cr release assay (Fig. 3).
              Three different cell lines were used as target
cells: 15-12 cells, 3T3 18 Neo BALB/c cells and P815 cells.

15-12 cells are BALB/c 3T3 fibroblasts transfected with a vector encoding *HIV-1* gp160 (Takahashi et al. <u>Proc. Natl. Acad. Sci. USA</u> <u>85</u>:3105 (1988)); 3T3 18 Neo BALB/c cells are BALB/c 3T3 fibroblasts transfected with an expression vector containing a Neor gene but no gp160 gene; and P815 cells are untransfected cells that present antigenic peptides added to the culture. CTL lysis of gp160 expressing 15-12 cells (closed squares in right panels) was compared to that of control gp160 non-expressing 3T3 18 Neo BALB/c cells (open diamonds right panels). P815 target cells (left panel) were tested in the presence (closed squares) or absence (open diamonds) of P18IIIB-I10 peptide (SEQ ID NO:16) (1μM). The percent specific 51Cr release was calculated as described above. CTL induced by IR immunization killed target cells either endogenously expressing HIV-gp160 or pulsed with P18IIIB peptide (SEQ ID NO:15).

Example 4. CTL induced by IR immunization are IL-12 dependent.

BALB/c mice were immunized IR with 4 doses (on days 0, 7, 14 and 21) of CLUVAC PCLUS3-18IIIB (SEQ ID NO:2) (50μg/mouse per immunization) in combination with CT (1μg/mouse). One day before and one day after immunization with peptide mice were treated intraperitoneally (i.p.) with anti-IL-12 antibody (0.5 mg/per injection: 4mg/mouse total dose (Fig. 4, right panels) or were untreated (Fig. 4, left panels). On day 35, antigen-specific T cells were isolated from PP, LP and SP. Immune cells from SP, PP, or LP were cultured as described above. Cytolytic activity of CTL was measured (Fig. 4) as described above. For testing the peptide specificity of CTL, 51Cr labeled P815 targets were pulsed with peptide P18IIIB-I10 (SEQ ID NO:16) at the beginning of the assay (closed squares), or (as a control) left unpulsed i.e., without peptide (open diamonds). Induction of both mucosal and systemic CTL responses by IR immunization was IL-12-dependent, as shown by inhibition of induction of CTL in mice treated i.p. with anti-IL-12 antibody.

BALB/c mice were immunized IR on days 0, 7, 14 and
21 with either composition A containing the synthetic *HIV-1*
CLUVAC PCLUS3-18IIIB (SEQ ID NO:2) (50µg/mouse per
immunization), CT (10µg/mouse per immunization), and

5   recombinant IL-12 (1µg/mouse per immunization), or composition
B containing the synthetic *HIV-1* CLUVAC PCLUS3-18IIIB (SEQ ID
NO:2) (50µg/mouse per immunization), and CT (10µg/mouse per
immunization), on day 35, antigen-specific T cells were
isolated from PP and SP. Immune cells from PP (Fig. 9A) and

10   SP (Fig. 9B) were separately cultured and tested for antigen
specific CTL, as described above. Fig. 9A shows killing of
peptide P18IIIB-I10 (SEQ ID NO:16) pulsed target cells by
effector cells from mice immunized with composition A (i.e.,
antigen with IL-12) (open squares), and Fig. 9B shows killing

15   of the P18IIIB-I10 (SEQ ID NO:16) pulsed target cells by
effector cells from mice immunized with composition B (i.e.,
antigen without IL-12) (open diamonds). In both Figs. 9A and
9B, control data was obtained using the two effector
populations tested on unpulsed targets. The enhanced CTL

20   responses of both PP (Fig. 9A) and SP (Fig. 9B) effector cells
from mice immunized IR with composition A compared to the
responses elicited by IR immunization with composition B
indicate that co-administration of IL-12 augments mucosal and
systemic CTL responses. These data both provide independent

25   evidence for the role of IL-12 in eliciting mucosal (and
systemic) immunity by mucosal administration of antigens and
confirm the findings of the antibody inhibition data described
above.

30   **Example 5. Intrarectal peptide immunization protects against
mucosal challenge with HIV-gp160 expressing recombinant
vaccinia virus.**

Groups of 5 mice were immunized IR with 4 doses of
the *HIV-1* CLUVAC PCLUS3-18IIIB (SEQ ID NO:2) (50µg/mouse per

35   immunization) on days 0, 7, 14 and 21 in combination with CT.
On day 35, mice were challenged IR with 2.5 x 10⁷ pfU of
vaccinia virus expressing gp160IIIB (vPE16). After 6 days,
the mice were killed and their ovaries assayed for the

presence of virus (Fig. 5) as described above. The left bar
of Fig. 5 shows virus titer in the ovaries of unimmunized mice
and the right bar shows virus titer in the ovaries of
immunized mice.   IR immunization with PLCUS3-18IIIB (SEQ ID
5   NO:2) protected mice against IR challenge with vPE16 as shown
by a 4.5-log reduction in viral pfu in ovaries compared to
unimmunized animals (p<0.005).

        Example 6. Comparison of systemic CTL responses six months
10   **after IR and IN immunization**
             Mice were immunized IR or IN with 4 doses (on days
        0, 7, 14 and 21) of the *HIV-1* CLUVAC PCLUS3-18IIIB (SEQ ID
        NO:2) (50μg/mouse). Six months after the first dose, antigen-
        specific T cells were isolated from SP and tested for the
15   presence of *HIV-1* P18IIIB-I10 (SEQ ID NO:16) peptide specific
        CTL (Fig. 6A and 6B) as described above. Closed squares show
        killing of P18IIIB-I10 (SEQ ID NO:16)-pulsed targets and open
        diamonds show killing of unpulsed targets. The level of CTL
        activity induced by IR immunization (Fig. 6A) was higher than
20   that induced by IN immunization (Fig. 6B). These data
        indicate that IR immunization induced potent, long-lasting,
        antigen-specific splenic immune responses.
             To evaluate different routes of immunization, mice
        were immunized mucosally (IR, IN, IG) or systemically (SC)
25   with 4 doses (on days 0, 7, 14 and 21) of the HIV-1 CLUVAC
        PCLUS3-18IIIB (SEQ ID NO:2) (50μg/mouse). Thirty-five days
        after the first dose, antigen-specific T cells were isolated
        from PP and SP and tested for the presence of *HIV-1* P18IIIB--
        I10 (SEQ ID NO:16) peptide-specific CTL as described above
30   (Fig. 7). Closed bars show killing of P18IIIR-I10 (SEQ ID
        NO:16) pulsed targets and open bars show killing on unpulsed
        targets. The level of CTL activity induced by IR immunization
        was significantly higher in inductive mucosal tissue (PP) and
        much higher in systemic immunological tissue (SP) than that
35   induced by the other mucosal routes (IN and IG). CTL activity
        was detected in both PP and SP after immunization via all
        three mucosal routes. Systemic immunization (SC) only
        resulted in significant CTL activity in SP.

Example 7. CTL induced by IR immunization
are IFNγ dependent.

Wild-type BALB/c mice and BALB/c mice lacking the
ability to produce functional IFNγ (IFNγ$^{-/-}$ mice) (Dalton et
al., _Science_ 259:1739 (1993); Tishon et al., _Virology_ 212:244
(1995)) were immunized IR with 4 doses (on days 0, 7, 14 and
21) of CLUVAC PCLUS3-18IIIB (SEQ ID NO:2) (50μg/mouse per
immunization) in combination with CT (1μg/mouse). On day 35,
antigen-specific T cells were isolated from PP, LP and SP.
Immune cells from SP, PP, or LP of wild type BALB/c and IFNγ$^{-/-}$
mice were cultured as described above. Cytolytic activity of
CTL was measured (Fig. 8A and Fig. 8B) as described above.
The peptide specificity of CTL was tested as follows: ^{51}Cr
labeled P815 targets were pulsed with peptide P18IIIB-I10 (SEQ
ID NO:16) at the beginning of the assay (solid bars), or (as a
control) left unpulsed i.e., without peptide (open bars).
Induction of both mucosal and systemic CTL responses by IR
immunization was IFNγ dependent, as shown by the lack of
detectable CTL activity in SP, PP, and LP cells from IFNγ$^{-/-}$
mice (Fig. 8B) and potent CTL activity in SP, PP, and LP cells
from wild-type BALB/c mice (Fig. 8A).

Summarizing the foregoing results, mucosal,
administration of an antigenic peptide to mice results in the
induction of a protective CTL response detectable in both the
inductive (Peyer's patch (PP)) and effector (lamina propria
(LP)) sites of the intestinal mucosal immune system, as well
as in systemic lymphoid tissue, i.e., spleen (SP). The
mucosal CTL response is enhanced by co-administering the
mucosal adjuvant, cholera toxin (CT) with the antigenic
peptide, is inhibited by antibody that specifically binds to
(thereby neutralizing the activity of) interleukin-12 (IL-12),
and is not detectable in mice lacking the ability to produce
functional interferon-γ (IFNγ). Furthermore, including IL-12
in the composition of antigenic peptide and CT used for IR
immunization resulted in enhanced PP and SP CTL responses
relative to those obtained by IR immunization with antigenic
peptide, CT and no IL-12. IR immunization with the viral

peptide resulted in protection from viral infection upon
subsequent IR challenge with the appropriate virus.

Example 8. Comparison of different HIV vaccine peptides for use in eliciting viral mucosal protection

Because of the variability of the V3 loop of HIV,
further studies were conducted comparing two cluster peptide
constructs using the V3 loop and incorporating the CTL epitope
P18 from strain IIIB (Ratner et al., Nature 313:277-284,
(1985)) or MN (Gurgo et al., Virology 164:531-536, (1998)) of
HIV-1. These studies were conducted as an exemplary analysis
to demonstrate that HIV vaccine cluster peptide constructs can
be prepared from different HIV strains and screened in side-
by-side assays to optimize induction of mucosal CTL immunity.

Animals For each of the following Examples, female
BALB/c mice were purchased from Frederick Cancer Research
Center (Frederick, MD). IFN-$\gamma^{-/-}$ mice were purchased from
Jackson Laboratories (Bar Harbor, ME).   Mice used in this
study were 6-12 weeks old. The IFN-$\gamma^{-/-}$ mice were maintained
in a specific pathogen-free microisolator environment.

Immunization Mice were immunized with 4 doses of
the synthetic HIV peptide vaccine construct PCLUS3-18IIIB
(Ahlers et al., J. Immunol. 150:5647-5665, (1993))
($50 \mu g$/mouse for each immunization) on days 0, 7, 14 and 21 in
combination with cholera toxin (CT) (10 μg/mouse) (List
Biological Laboratories, Campbell, CA) by intrarectal
administration. For subcutaneous immunization, incomplete
Freund's adjuvant was used. rm IL,-12 (a generous gift of
Genetics Institute, Inc., Cambridge, MA) was delivered either
intraperitoneally (IP) ($1 \mu g$) or intrarectally ($1 \mu g$) mixed with
DOTAP (Boehringer Mannheim) , a cationic lipofection agent,
along with the peptide vaccine.

Cell purification Five weeks to 6 months after the
first dose, antigen-specific T cells were isolated from
Peyer's patches (PP), lamina propria (LP) and the spleen (SP).
The Peyer's patches were carefully excised from the intestinal
wall and dissociated into single cells by use of collagenase
type VIII, 300U/ml (Sigma) as described, Mega et al., Int.

Immunol. 3:793-805, (1991). Our data showed that most PP CD3⁺
T cells isolated from normal mice were CD4⁺, while CD3⁺CD8⁺ T
cells were less frequent. Further, collagenase did not alter
expression of CD3, CD4, or CD8 on splenic T cells treated with
this enzyme. Lamina propria lymphocyte (LPL) isolation was
performed as described, Mega et al., _Int. Immunol. 3_:793-805,
(1991). The small intestines were dissected from individual
mice and the mesenteric and connective tissues carefully
removed. Fecal material was flushed from the lumen with un-
supplemented medium (RPMI 1640). After the PP were identified
and removed from the intestinal wall, the intestines were
opened longitudinally, cut into short segments, and washed
extensively in RPMI containing 2% fetal bovine serum (FBS).
To remove the epithelial cell layer, tissues were placed into
100ml of 1 mM EDTA and incubated twice (first for 40 min and
then for 20 min) at 37°C with stirring. After the EDTA
treatment, tissues were washed in complete RPMI medium for 10
min at room temperature and then placed into 50 ml of RPMI
containing 10% FCS and incubated for 15 min at 37° with
stirring. The tissues and medium were transferred to a 50 ml
tube and shaken vigorously for 15 seconds, and then the medium
containing epithelial cells was removed. This mechanical
removal of cells was repeated twice more, using fresh medium
each time, in order to completely remove the epithelial cell
layer. Histologic examination revealed that the structure of
the villi and lamina propria were preserved. To isolate LPL,
tissues were cut into small pieces and incubated in RPMI 1640
containing collagenase type VIII 300 U/ml (Sigma) for 50 min
at 37°C with stirring. Supernatants containing cells were
collected, washed and then re-suspended in complete RPMI 1640.
This collagenase dissociation procedure was repeated two times
and the isolated cells pooled and washed again. Cells were
passed through a cotton-glass wool column to remove dead cells
and tissue debris and then layered onto a discontinuous
gradient containing 75% and 40% Percoll (Pharmacia Fine
Chemicals, Pharmacia Inc., Sweden). After centrifugation
(4°C, 600g, 20min), the interface layer between the 75% and
40% Percoll was carefully removed and washed with incomplete

medium. This procedure provided >90% viable lymphocytes with a cell yield of 1.5-2 X 10^6 lymphocytes/mouse. The SP were aseptically removed and single cell suspensions prepared by gently teasing them through sterile screens. The erythrocytes were lysed in Tris-buffered ammonium chloride and the remaining cells washed extensively in RPMI 1640 containing 20/o FBS.

Cytotoxic T lymphocyte assay   Immune cells from SP, PP, LP were cultured at $5X10^6$ per/milliliter in 24-well culture plates in complete T cell medium (CTM): RPMI 1640 containing 10% FBS, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 5×10^{-5} M 2-mercaptoethanol. Three days later we added 10% concanavalin A supernatant-containing medium as a source of IL-2. LPL were studied after 7 days stimulation with IμM P18IIIB-I10 peptide together with $4X10^6$ of 3300-rad irradiated syngeneic spleen cells. SP and PP cells were stimulated in vitro similarly for one or two 7-day culture periods before assay. Cytolytic activity of CTL lines was measured by a 4-hour assay with ^{51}Cr labeled targets. Two different cell lines were used as a target cells: 1) 15-12 cells, Takahashi et al., Proc. Natl. Acad. Sci. USA 85:3105-3109 (1988) (BALB/c 3T3 fibroblasts transfected with HIV-1IIIB gp160 and endogenously expressing HIV gp160), compared with 18 Neo BALB/c 3T3 fibroblasts transfected with Neo^R alone as a control, and 2) P815 targets tested in the presence or absence of I10 peptide (IμM). For testing the peptide specificity of CTL, ^{51}Cr labeled P815 targets were pulsed for 2 hours with peptide at the beginning of the assay. The percent specific ^{51}Cr release was calculated as 100 X (experimental release-spontaneous release)/(Maximum release - spontaneous release). Maximum release was determined from supernatants of cells that were lysed by addition of 5% Triton-X 100. Spontaneous release was determined from target cells incubated without added effector cells.

Vaccinia virus   Recombinant vaccinia virus vPE16 expresses the HIV-1 gp160 gene from isolate IIIB (BH8) (Earl et al., J. Virol. 64:2448-2451 (1990)). Expression is directed by the compound early/late P7.5 promoter. Two copies

of the sequence T5NT, which serves as a transcription
termination signal for early vaccinia virus genes, are present
in the IIIB gp 160 gene. Both of these have been altered in
vPE16, so as to retain the original coding sequence and allow
early transcription of the gene. The virus, vSC8, is used as
a negative control without gp160 (Chakrabarti et al., <u>Mol.</u>
<u>Cell Biol.</u> 5:3403-3409 (1985)). Both vPE16 and VSC8 express
beta-galactosidase.

        <u>Determination of virus titer in the ovary</u> On day
35 or 6 months after cluster peptide HIV vaccine immunization,
mice were challenged intrarectally with 2.5 X 10^7 or 5 X 10^7
pfu of vaccinia virus expressing gp160IIIB (vPE16). Six days
after the challenge with recombinant vaccinia virus expressing
HIV-gp160, the mice were killed and ovaries were removed,
homogenized, sonicated, and assayed for vPE16 titer by plating
serial 10-fold dilutions on a plate of BSC-2 indicator cells
staining with crystal violet and counting plaques at each
dilution. The minimal detectable level of virus was 100 pfu.

        To compare different HIV vaccine cluster peptide
constructs, BALB/c mice were immunized intrarectally with
peptide (PCLUS3-18IIIB or PCLUS3-18MN) in the presence of CT
as a mucosal adjuvant weekly for four weeks (on days 0, 7, 14,
and 21). Mice were studied either two weeks later (day 35) or
at six months for memory CTL responses in the Peyer's Patches
(PP) or_spleen (SP). IR immunization with both peptides
PCLUS3-18IIIB or PCLUS3-18MN in combination with CT induces a
P18-specific CTL response in the intestinal PP (Fig. 10, panel
A) and in the spleen (Fig. 10, panel B).

        However, the level of CTL response after IR
immunization with PCLUS3-18MN was significantly lower than
after IR immunization with PCLUS3-18IIIB. The difference may
reflect a higher affinity of the minimal 10-mer P18IIIB-I10,
compared to P18MN-T10, for H-2D^d (Takahashi et al., <u>Science</u>
246:118-121 (1989); Takeshita et al., <u>J. Immunol. 154</u>:1973-
1986 (1995)). Also, much higher production of IFN-γ by
mucosal P18IIIB-specific CD8$^+$ CTL was observed compared to
P18-MN-specific CTL after stimulation with specific peptide <i>in</i>
<i>vitro</i> for 48 hours. When tested on 15-12 gp160IIIB-

transfected fibroblast targets endogenously expressing gp
160IIIB, the CTL elicited by immunization with PCLUS3-18IIIB
also killed these targets. On the basis of these
observations, the PCLUS3-18IIIB construct was used in the
5   protection experiments described below.

Example 9. Mucosal immunization of mice with cluster peptide
construct provides longlasting protection from infection with
recombinant vaccinia virus expressing HIVg160
10   In the foregoing Examples, the ability of the
mucosal immune responses induced by the HIV cluster peptide
vaccine to protect against virus challenge via a mucosal route
is demonstrated. To determine the specificity of this
protection for recombinant protein HIV-1 IIIB gp160, IR
15   immunized mice were challenged on day 35 after the start of
immunization by IR infusion with vaccinia virus expressing
HIV-1 IIIB gp 160 (vPE16), or with control vaccinia virus
expressing β-galactosidase (vSC8). Unimmunized animals
challenged with vPE16 or vSC8 served as controls. Six days
20   after the challenge, mice were sacrificed and the ovaries were
removed and assayed for vaccinia titer (6 days after infection
with vaccinia, the ovaries contain the highest titer of
virus).
              IR immunization with the synthetic HIV peptide
25   vaccine protected mice against an IR challenge with vaccinia
virus expressing HIV-1 IIIB gp160 compared to unimmunized
controls, but did not protect against IR challenge with
vaccinia virus expressing only an unrelated protein, β-
galactosidase (Fig. 11). Thus, the protection was specific
30   for virus expressing HIV-1 gp160, and any nonspecific
inflammatory response induced by the peptide infusion
intrarectally was not sufficient to protect against viral
challenge two weeks after the last dose of the immunization.
              Although the presence of mucosal memory CTL
35   precursors was observed, requiring restimulation in vitro for
activity 6 months after IR immunization (Belyakov et al.,
Proc. Nati. Acad. Sci. 95:1709-1714 (1998)), the strength and
duration of protection remained unclear. To resolve this

question, the IR immunized mice were challenged 6 months after the start of immunization with PCLUS3-18IIIB by IR administration with vaccinia virus expressing HIV-1 IIIB (vPE16). This study showed that, even 6 months after HIV

5   cluster peptide immunization, BALB/c mice exhibit protection against recombinant HIV-vaccinia challenge (Fig. 12).

        Example 10. Protection of mice against mucosal viral
        challenge is mediated by CD8⁺ CTL in the mucosal site

10   To determine the immune mechanism responsible for protection against mucosal challenge with virus expressing HIV gp160, mice were treated IP with 0.5 mg monoclonal anti-CD8 antibody (clone 2.43, NIH, Frederick, MD) one day before and after each of the four immunizations and also two days before

15   and three days after the challenge with vPE16. This treatment led to a significant inhibition of the protection against mucosal challenge with vPE16 (Fig. 13). Thus, protection in the mucosal site against the virus expressing HIV-1 gp160 is mediated by CD8⁺ lymphocytes.

20   Because the HIV peptide constructs disclosed herein elicit both strong mucosal and systemic MHC class I restricted CD8⁺ CTL responses (Belyakov et al., <u>Proc. Nati. Acad. Sci.</u> <u>95</u>:1709-1714 (1998)), the role of these responses in mediating protection were further investigated. Because SC immunization

25   with peptide vaccine elicits splenic but not mucosal CTL, whereas IR immunization elicits both (Fig. 14), SC and IR immunizations can be compared to determine whether systemic CTL are sufficient to protect against mucosal challenge, or whether local mucosal CTL are necessary. Accordingly, mice

30   were immunized with PCLUS3-18IIIB plus IFA by the SC route or with PCLUS3-18IIIB and CT by the IR route on days 0, 7, 14 and 21, and compared these. On day 35 after the start of immunization, these groups of mice as well as unimmunized control mice were challenged by IR administration of vaccinia

35   virus expressing HIV-1 gp160 (vPE16). Finally, six days after the challenge, mice were sacrificed and their ovaries assayed for viral titer.

SC immunization with PCLUS3-18IIIB did not protect mice against mucosal challenge with vPE16, whereas IR immunization with the same peptide did protect (Fig. 14B). Thus, protection against mucosal challenge with virus

5   expressing HIV-1 gp160 can be induced only by mucosal immunization of mice, and correlates with local mucosal CTL activity, not with splenic CTL activity. On this basis one can conclude that the CD8⁺ CTL-mediated protection from mucosal challenge with recombinant vaccinia expressing HIV-1

10   gp160 requires local mucosal CD8⁺ CTL, whereas a systemic CTL response is not sufficient.

Example 11. Cytokine dependence and enhancement of protection by local administration of IL-12 with the vaccine

15   Induction of mucosal CTL by peptide vaccine is dependent on endogenous IL-12 in that it can be blocked by *in vivo* treatment of mice with anti-IL-12 (Belyakov et al., <u>Proc. Nati. Acad. Sci. 95</u>:1709-1714, (1998)). To further define the role of IL-12 in the CTL response and protection, BALB/c (H-

20   2Dᵈ) mice were treated by the IP route with 1 μg of the rmIL-12 each day of the IR immunization with PCLUS3-18IIIB (50 μg/mice). This treatment did not lead to significant changes in the HIV-specific CTL activity in either mucosal or systemic sites (Fig. 15). However, when the mice were treated with the

25   rmIL-12 (1 μg) + DOTAP intrarectally together with peptide, we found a significant increase in the CTL level in both mucosal and systemic sites 35 days after the start of immunization (Fig. 15).

                 In view of the above results, the possibility that

30   rmIL-12 administered at the local site and time of mucosal immunization might increase protection against mucosal challenge with vaccinia virus expressing HIV-1 gp160 was investigated. To address this question, BALB/c mice were immunized with the rmIL-12 + DOTAP intrarectally together with

35   peptide. The mice were then challenged mice on day 35 after the start of immunization by IR administration of vaccinia virus expressing HIV-1IIIB gp160. In this study, twice the dose of challenge virus was used, whereby the unimmunized mice

had a titer of several times 10^{10} rather than several times 10^8 seen in the previous Examples using a lower challenge dose. Nevertheless, the immunized mice showed a reduction of greater than 4 logs in virus titer, as had been seen in the earlier experiments (Fig. 16, bar 2).

Importantly, IR immunization with the synthetic HIV peptide vaccine plus rmIL-12 protected mice against an IR challenge with this gp-160-recombinant vaccinia virus even more effectively than after the IR immunization with peptide alone (6-log reduction in viral pfu versus 4-log reduction, $p < 0.05$) (Fig. 16, bar 3 versus bar 2).

As the induction of mucosal $CD8^+$ CTL is strongly dependent on IL-12 and IFN-γ (Belyakov et al., _Proc. Natl. Acad. Sci._ _95_:1709-1714 (1998)), further studies were undertaken herein to determine which cytokine acts directly for the generation of mucosal CTL, and which acts through a secondary mechanism. To address this question, IFN$\gamma^{-/-}$ mice (BALB/c background) and conventional BALB/c mice were treated with the rmIL-12 (I μg/mouse) + DOTAP IR together with peptide. Mucosal treatment of IR-immunized IFN$\gamma^{-/-}$ mice with rmIL-12 did not lead to the induction of mucosal or systemic CTL (Fig. 17). It thus appears that IL-12 cannot act directly in the induction of mucosal $CD8^+$ CTL in the absence of IFNγ.

In summary, the foregoing Examples incorporate a novel viral challenge system in which recombinant vaccinia virus expressing HIV-1 gp160 is used as a surrogate for HIV-1, since we cannot infect the mice with HIV-1. Importantly, in this system, neutralizing antibodies to gp160 cannot protect against recombinant vaccinia expressing gp160, because the virus does not incorporate gp160 in the virus particle but expresses it only in the infected cell. Thus, the protective immune response must be directed at the infected cell.

The results herein demonstrate that the protective response is completely dependent on $CD8^+$ cells., by the abrogation of protection after _in vivo_ depletion of $CD8^+$ cells. Thus, the results show unequivocally that it is $CD8^+$ CTL (whether via lytic activity or via secretion of cytokines or other soluble factors) that protect. Since it has been

shown that protection against vaccinia infection can be mediated by interferon-γ which is secreted by CD8⁺ CTL in response to antigen stimulation (Harris et al., <u>J. Virol.</u> <u>69</u>:910-915 (1995)), it is possible that the mechanism involves local secretion of this cytokine by the CTL rather than lysis of infected cell. By either mechanism, the CTL are responsible for mediating protection.

However, since the mucosal immunization induces CTL in both the local mucosal site and the spleen, this result does not distinguish which CTL are responsible for protection. To address this important question, the present Examples take advantage of the fact that subcutaneous immunization with the peptide vaccine induces systemic CTL in the spleen at a level at least as high as that induced by mucosal immunization, but does not induce mucosal CTL. Splenic CTL resulting from both immunization routes kill target cells endogenously expressing HIV-1 gp160. Thus, if systemic CTL against this epitope protected against mucosal challenge, then the subcutaneously immunized mice would have been expected to be protected.

However, the subcutaneously immunized mice showed no evidence of protection against mucosal challenge. Thus, the protection correlated with CTL activity in the local mucosal sites, Peyer's patches and lamina propria, not with CTL activity in the spleen. The protection was not only mediated by CTL, but also required CTL in the local mucosal site of challenge. Systemic CTL were not sufficient.

Protection mediated by local mucosal CTL appears independently sufficient to mediate a protective immune response. This conclusion is supported by the observation that a two-log reduction in viral pfu occurs in the ovary even at day 2 after mucosal viral challenge (from 2.37×10^6 pfu in unimmunized mice to 3.34×10^4 pfu in IR immunized mice), before much replication could have occurred in the ovary. This finding indicates that the reduction in titer in the ovary reflects a reduction in the amount of virus that can escape the initial mucosal site of infection. In addition, the enhancement of CTL activity and protection by rmIL-12

depended on local mucosal administration of the cytokine, not
systemic administration.

　　　　　One possible difference between CTL induced by
mucosal versus systemic immunization is that the CTL resulting
5　　from the SC immunization do not have homing receptors for the
GI mucosa, as evidenced by the fact that they are note
detected in the lamina propria or Peyer's patches.

　　　　　The present disclosure represents the first
demonstration of protection against GI mucosal challenge
10　　requiring local mucosal CTL at the site of challenge. These
results have important implications for the development of
protective vaccines against mucosal exposure to viruses.

　　　　　In addition to these results, the Examples provided
herein also demonstrate a surprising persistence, not only of
15　　memory CTL in the mucosa, but also of protective immunity
against mucosal viral challenge. Factors controlling CTL
memory, and the role of persistent antigen in maintaining
memory CTL, represent another issue that has been of interest
for some time (Ahmed and Gray, Science 272:54-60 (1996);
20　　Kundig et al., Proc. Natl. Acad. Sci. U.S.A. 93:9716-9723
(1996); Ehl et al., Eur. J. Immunol. 27:3404-3413 (1997)), but
has been little studied in the context of mucosal immune
responses and protection. In studies of systemic immunity, it
was shown (Slifka et al., Blood 90:2103-2108, (1997)) that
25　　after infection with lymphocytic choriomeningitis virus
(LCMV), CTL memory responses were present in the bone marrow
for at least 325 days, indicating long-term persistence of
antiviral T cells at this site. While the antigen-specific
CD8$^+$ T cell number dropped precipitously following viral
30　　clearance, substantial numbers persisted for the life of the
mouse (Muraii-Krishna et al., Immunity 8:177-187, (1998)).
Upon rechallenge with LCMV, there was rapid expansion of
memory CD8$^+$ T cells.

　　　　　These results indicate that systemic infection with
35　　virus can lead to long-term memory and protection. In the
case of mucosal CTL memory, it was shown that memory CTL
remained at the mucosal site longer if the immunization was
via the mucosal route (Gallichan and Rosenthal, J. Exp. Med.

<u>184</u>:1879-1890 (1996)), but the duration of protection by such mucosal CTL was not studied. The ability of mucosal memory CTL to protect will depend in part on the rapidity by which they can expand and be activated after virus exposure, which may relate to the level of virus replication in the mucosal site. For these reasons, it is important to determine the duration of mucosal protection dependent on local mucosal CD8$^+$ CTL. The results herein show persistence of CTL even six months after mucosal immunization with the peptide vaccine construct, without additional reimmunization beyond the initial three-week course. This response is accompanied by protection against mucosal challenge with vaccinia virus expressing gp160.

This latter result is particularly striking because the exemplary immunogen is a peptide administered without any depot form of adjuvant that would maintain the presence of antigen for extended periods. It would be expected that free peptide delivered to the lumen of the gut, or even after transport by mucosal cells, would have a very short half-life. Therefore, either memory CTL can persist in the mucosa at levels sufficient to mediate protection in the absence of persistent antigen, or antigen must persist locally in some cell-bound form, perhaps on N4HC molecules of dendritic cells. Yet another possibility is that the peptide crossreacts with one of the antigens in the mucosal flora and that these crossreactive antigens maintain the memory CTL.

Protection against viral infection by CTL involves more than just the number of CTL induced. Quality of CTLs is as important for *in vivo* protection as quantity. Previous reports have shown that high-avidity P18IIIB-specific CTLs adoptively transferred into severe combined immunodeficient (SCID) mice were 100- to 1000-fold more effective at viral clearance than the low-avidity CTLs specific for the same peptide-MHC complex, despite the fact that all CTL lines lysed virus infected targets *in vitro* (Alexander-Miller et al., <u>Proc. Natl. Acad. Sci. U.S.A. 93</u>:4102-4107 (1996)). Thus, the CTL induced by mucosal immunization with the synthetic peptide

vaccine must be present not only in sufficient quantity to protect, but also must be of high enough avidity to protect.

Other aspects of the present disclosure enable optimization of induction of the CTL response and protective immunity. Delivery of certain cytokines such as IL-12 at the site of antigen immunization systemically have been shown to enhance systemic CTL responses (Ahlers et al., _J. Immunol. 159_:3947-3958 (1997); Iwasaki et al., _J. Immunol. 159_:4591-4601 (1997); Xiang and Ertl, _Immunity 2_:129-135 (1995); Irvine et al., _J. Immunol. 156_:238-245 (1996)). However, no comparable studies have been conducted for mucosal CTL responses.

In the model system disclosed herein, induction of mucosal CTL is dependent on endogenous production of IL-12 by the mouse, because it could be inhibited by anti-IL-12 antibody given _in vivo_ before and after each immunization. In the present Examples, IL-12 co-administered with the antigen intrarectally significantly enhanced CTL induction, and also increased protection against intrarectal vaccinia viral challenge. However, it was striking that IL-12 delivered systemically (i.p.) did not enhance CTL induction either in the systemic sites (eg. spleen) or in the mucosa. This difference may be due to the short half life of IL-12 delivered systemically, which prevented it from surviving long enough to get to the sites of CTL induction. Therefore, for mucosal CTL induction as well as for systemic CTL induction, it is important to deliver the cytokine directly to the site of antigen administration and CTL induction.

Enhancement of CTL induction in the mucosa with recombinant IL-12 is a useful strategy for mucosal vaccine development. In this context, small doses given locally in the mucosal sites are not likely to have the global toxicity that has been associated with systemic administration of this cytokine.

The results herein further show that enhancement of the CTL response _in vivo_ by rmIL-12 is dependent on interferon-γ, as no enhancement was observed in IFNγ$^{-/-}$ mice. At least two mechanisms can explain this result. First, IL-12

may be acting through its well-defined ability to induce production of IFN-γ (Trinchieri, G., Blood 94:4008-4027 (1994)), which then acts directly on CTL precursors. Alternatively, since IFN-γ is important for expression of the IL-12 receptor (Szabo, et al., J. Exp. Med. 185:817-824 (1997)) IL-12 may act directly on CTL, but may not be able to act in IFN$\gamma^{-/-}$ mice because of the lack of IL-12R expression.

There is evidence that CTL activity may play a role in protective immunity in humans against HIV-1 (reviewed in (Rowland-Jones et al., Adv. Immunol. 65:277-346 (1997); Yang, O. O. and B. D. Walker, Adv. Immunol. 66:273-311 (1997); Berzofsky and Berkower, AIDS 9(A):S 143-S 157 (1995)). Cell-mediated immunity to HIV-1 has been demonstrated in uninfected high risk adults (Pinto et al., J. Clin. Invest. 96:867-876, (1995); Rowland-Jones et al., Nature Medicine 1:59-64, (1995)) and in uninfected children born to infected mothers (Clerici et al., AIDS 7:1427-1433 (1993); Luzuriaga and Sullivan, J. Cell. Biochem. Supplement 17E:98.(Abstract) (1993)). CTL activity has been correlated with low viral load and long-term non-progressor status in some infected individuals (Cao et al., N. Engl. J. Med. 332:201-208 (1995)). CTL have also been associated with recovery from acute HIV or SIV infection (Yasutomi et al., J. Virol. 67:1707-1711 (1993); Reimann et al., J. Virol. 68:2362-2370 (1994); Koup et al. J. Virol. 68:4650-4655 (1994); Borrow et al., Nature Medicine 3:205-211, (1997)). Induction of escape mutations by CTL implies that the CTL are eliminating the bulk of the wild type virus (Borrow et al., Nature Medicine 3:205-211 (1997); Phillips et al., Nature 354:453-459 (1991); Nowak et al., Nature 375:606-611 (1995); Goulder et al., Nature Medicine 3:212-217 (1997); Couillin et al., J. Exp. Med. 180:1129-1134 (1994); Koenig et al., Nature Medicine 1:330-336 (1995)). In addition, in an SIV study, protection was associated with a particular simian class I MHC molecule (Heeney et al., J. Exp. Med. 180:769-774, (1994)). CD8⁺ cells have been demonstrated to suppress replication of human and simian lentiviruses in autologous CD4 cells by a non-lytic mechanism involving soluble factors synthesized and released by activated CD8⁺ cells (Walker et

al., _Science 234_:1563-1566 (1986); Tsubota et al., _J. Exp._
Med. 169:1421-1434 (1989); Walker and Levy, _Immunology 66_:628-
630 (1989); Mackewicz et al., _J. Clin. Invest. 87_:1462-1466,
(1991); van Kuyk et al., _J. Immunol. 153_:4826-4833 (1994)).

Such soluble factors include the chemokines RANTES, MIP-1α,
and MIP-1β (Cocchi et al., _Science 270_:1811-1815 (1995)).

The present disclosure provides the first
demonstration of protection against mucosal viral challenge
mediated by CTL, which must be present in the local mucosa.
Also provided are methods and compositions for enhancing the
induction of such local mucosal CTL by a peptide vaccine given
together with recombinant IL-12 at the mucosal site. These
methods and compositions for stimulating CTL immunity at local
sites of mucosal exposure represent important tools for
vaccine development to prevent HIV infection or disease in
humans. Because the gastrointestinal tract appears to be a
major site of early SIV and HIV replication, among other
pathogens, and because this and other mucosal sites are
frequent sites of entry for these pathogens, it is
particularly critical to achieve protection at mucosal sites.
The present disclosure satisfies these objects and provides
other advantages as set forth above.

Although the invention has been described with
reference to the presently preferred embodiment, it should be
understood that various modifications can be made without
departing from the spirit of the invention. Accordingly, the
invention is limited only by the following claims.